PATENT SPECIFICATION

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(54) CLAVAM DERIVATIVES

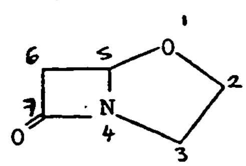
(71) We, GLAXO LABORATORIES LIMITED, a British Company of Greenford, Middlesex, England, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to novel antibiotic compounds and to a process for their production.

Fermentation of *Streptomyces clavuligerus*, and in particular strain NRRL 3585, is known to produce a number of antibiotic substances and British Patent Specification No. 1,315,177 describes and claims the cultivation of *Streptomyces clavuligerus* strain NRRL 3585 until a substantial amount of two β-lactam carboxylic acids, referred to as Antibiotics A 16886 I and A 16886 II is produced. In German OLS 2,604,697 we have described the isolation from such fermentation broths of a further β-lactam carboxylic acid, clavulanic acid.

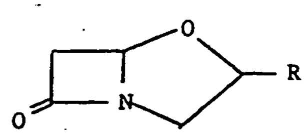
We have now been able to isolate from fermentations of strains of *Sterptomyces clavuligerus* further β-lactam compounds which have been found to possess antibiotic activity.

The compounds of this specification are named with reference to "clavam", the name given to the parent heterocycle of formula A



by analogy with the term "cepham" used in the naming of cephalosporin compounds in J.Amer.Chem.Soc., 1962, *84*, 3400.

According to one aspect of this invention, therefore we provide a compound of the formula (I)



(I)

wherein R represents a hydroxymethyl group, said compound in deuteroacetone exhibiting the 100 MHz proton n.m.r. τ values shown in Table 1 herein, and the corresponding compounds in which R is a formyloxymethyl group or a carboxyl or

esterified carboxyl group and, in the case where R represents a carboxyl group, salts thereof.

TABLE I

τ value	τ value
4.68 (d, 2.5 Hz) (1H)	7.09 (dd, 6 Hz and 11.5 Hz) (1H)
5.58 (multiplet) (1H)	6.32 (broad singlet) (2H)
5.77 (broad singlet, exchanges with D_2O) (1H)	6.72 (dd, 2.5 Hz and 16 Hz) (1H)
6.14 (dd, 7 Hz and 11.5 Hz) (1H)	7.29 (d, 16 Hz) (1H)

It is believed that the τ values given in Table 1 and in Tables 2 and 3 hereinafter are subject to an experimental error of 0.05.

It should be noted that although the stereochemical configuration of the compounds of the invention is not known, the stated τ values in Table I are characteristic of the particular configuration existing in the compound of formula (I) in which R is hydroxymethyl and all the compounds have the same configuration at the 2 and 5 positions.

Compounds of formula (I) in which R is hydroxymethyl, formyloxymethyl or esterified carboxyl show useful and antifungal activity; the compounds in which R is carboxy and salts thereof are primarily of use in preparing active esters.

The salts according to the invention include salts with inorganic bases, such as alkali metal salts, e.g. sodium, potassium and lithium salts; alkaline earth metal salts, e.g. calcium and magnesium salts; and ammonium salts, as well as salts with organic bases, for example amine salts.

The esters according to the invention may be represented as compounds of formula I in which R is a group —COOR' where R' represents an organic group which is conveniently derived from an alcohol (aliphatic or araliphatic) or a phenol. Such an alcohol or phenol used to esterify the carboxyl group preferably contains not more than 24 carbon atoms. While in general possessing antifungal activity, those esters according to the invention which are readily cleaved, for example by hydrogenolysis, are also of use as protected forms of the parent acid, for example in the preparation of further esters.

Thus, the group R' may represent a straight or branched unsubstituted or substituted alkyl or alkenyl group, preferably having the 1—8 carbon atoms, for example, a methyl, ethyl, propyl or isopropyl, butyl, sec-butyl tert-butyl or allyl group, desirable substituents being, for example, alkoxy, e.g. methoxy; halogen, i.e. fluorine, chlorine, bromine or iodine; cyano; acyloxy, e.g. alkanoyloxy, such as acetoxy or pivaloyloxy, or alkoxycarbonyloxy, such as ethoxycarbonyloxy; acyl, e.g. *p*-bromobenzoyl; and alkoxycarbonyl, e.g. ethoxycarbonyl;

an aralkyl group having up to 20 carbon atoms, especially an arylmethyl group, e.g. a benzyl or substitued benzyl group, suitable substituents being either halo, e.g. chloro; nitro, e.g. *o* or *p*-nitro; cyano; alkoxy, e.g. *p*-methoxy, or alkyl e.g. *p*-methyl groups; a diphenylmethyl or triphenylmethyl group or a fur-2-ylmethyl, thien-2-ylmethyl or pyrid-4-ylmethyl group, the hetero-cyclic groups of which may also be substitued, e.g. by a C_{1-4} alkyl group, pereferably methyl;

an aryl group having up to 12 carbon atoms, e.g. a phenyl or substituted phenyl group, suitable substituents being halo, e.g. chloro; nitro e.g. *o*- or *p*-nitro; cyano; alkoxy, e.g. *p*-methoxy; or alkyl, e.g. *p*-methyl groups;

a cylcoalkyl group containing not more than 12 carbon atoms, e.g. adamantyl; or

a heterocyclic group containing not more than 12 carbon atoms, the hetero atom being, for example, oxygen, as in the tetrahydropyranyl or phthalidyl group.

The compound of formula (I) in which R is $—CH_2OH$ has a negative molecular rotation $[M]_D^{23}$ in dimethyl sulphoxide, namely −166°. The 100 MHz proton nmr spectrum of a solution of this compound in deuteroacetone revealed τ values as shown in Table I, the full spectrum being shown in Fig. 1 of the accompanying drawings.

The compound of formula (I) wherein R represents a formyloxymethyl group shows in deuterochloroform solution τ values in the 100 MHz proton nmr spectrum as shown in Table 2.

TABLE 2

τ values	τ values
1.92 (s) (1H)	4.68 (d, 2.5 Hz) (1H)
5.43 (multiplet) (1H)	5.76 (3, 4 Hz) (2H)
6.00 (dd, 7 Hz, 11.5 Hz) (1H)	7.20 (dd, 6 Hz, 11.5 Hz) (1H)
6.70 (dd, 2.5 Hz, 16 Hz) (1H)	7.19 (d, 16 Hz) (1H)

The compound of formula I in which R is a diphenylmethoxycarbonyl group shows in deuterochloroform solution the τ values in the 100 MHz proton nmr spectrum shown in Table 3. The molecular rotation $[M]_D^{23}$ of the compound in dimethylsulphoxide is negative, namely −352°.

TABLE 3

peak	
2.69 (s) (10H)	5.85 (dd, 8 Hz and 12 Hz) (1H)
3.08 (s) (1H)	6.94 (dd, 5 Hz and 12 Hz) (1H)
4.53 (d, 3 Hz) (1H)	6.71 (dd, 3 Hz and 16 Hz) (1H)
5.10 (dd, 5 Hz and 8 Hz) (1H)	7.15 (d, 16 Hz) (1H)

Esters according to the invention, e.g. the C_{1-4} alkyl esters, for example the methyl ester, and the compounds in which R is a hydroxymethyl or formyloxymethyl group have been found to possess antifungal activity, for example against strains of *Candida albicans*, *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, *Trichophyton mentagrophytes*, *Epidermophyton flocossum* and *Microspora canis*.

Accordingly, we further provide an antifungal composition which includes one or more antifungal compounds of formula I (i.e. compounds in which R is hydroxymethyl, formyloxymethyl or esterified carboxyl together with a suitable carrier or diluent.

The esters of the invention, for example the methyl ester, and the compounds in which R is a hydroxymethyl or formyloxymethyl group have further been found to have activity against a range of fungal plant pathogens, for example *Botrytis allii* (onion neck rot), *Cercospora melanis* (melon blotch), *Verticillium sp.* (tomato wilt), *Fusarium graminium* (cereal seedling blight), *Rhizoctonia solani* (potato black scurf), *Altenaria brassicicola* (cabbage black spot), *Colletotricum coccodes* (tomato anthracnose), *Nectria galligena* (apple canker), *Botrytis cinerea* (grey rot), *Ashbya gossypii* (cotton boll disease), *Eremothecium ashbyi* (cotton boll disease), and *Fusarium oxysporum* (fusarium wilt), and some insect pathogens, for example *Beauvaria bassiana*. The above methyl ester has also shown activity against the plant pathogen *Ophiobolus graminis* and the compound in which R is a hydroxymethyl group has also shown activity against the plant pathogens *Phoma betae* (sugar beet blackleg) and *Ustilago hordei* (barley smut) and the insect pathogen *Entomopthora virulenta*.

The compounds in which R is a hydroxymethyl or formyloxymethyl group have also shown activity against *Erysiphe graminis* (barley mildew).

The antifungal compounds according to the invention may be used in human and veterinary medicine in the form of pharmaceutical compositions containing one or more pharmaceutical carriers or excipients suitable, for example, for oral, topical, rectal, intravaginal or parenteral administration. Such compositions may be used together with other medicinal agents. The compositions may be formulated in conventional manner. Thus, for example formulations for external applications may be prepared in oily, aqueous or powdered media in the form of conventional skin paints, lotions, creams, ointments, aerosols or dusting powders.

The pharmaceutical compositions according to the invention preferably contain the active material at a concentration of 0.1 to 95% by weight, advantageously 0.5 to 40%.

For horticultural or agricultural use the antifungal compounds according to the invention may be formulated for use in any desired way. Generally such formulations will include the compound in association with a suitable carrier or diluent. Such carriers may be liquid or solid and designed to aid the application of the compound either by way of dispersing it where it is to be applied or to provide a formulation which can be made by the user into a dispersible preparation. Thus, the compounds of the invention may be formulated as, for example, dusts, powders, granulates, pellets, sprays, smokes and mists in conventional manner. In

these formulations, the concentration of active material is preferably between 0.1% and 40% by weight.

The antifungal compounds of the invention may also be of use as storage preservatives in certain materials, for example, food, wallpaper paste, paint or petrol, or in beer and wine to prevent undesirable fermentation. In addition, the compounds may be of use as seed dressings.

The compounds of the invention may be isolated from a fermentation broth prepared by culture of a strain of *Streptomyces clavuligerus*.

Particularly useful strains are *Streptomyces clavuligerus* strain NRRL 3585 and mutants thereof. We have found strains NCIB 11260 and NCIB 11261 to be especially useful. Strain NCIB 11260 is a single colony isolate from strain NRRL 3585 having essentially similar morphology to NRRL 3585, as described in British Patent Specification No. 1,315,177. Strain NCIB 11261 also has essentially similar morphology to strain NRRL 3585, except that it requires uracil for growth.

As used herein, the term 'mutant' will include any mutant strain which arises either spontaneously or as a result of the action of an external agent, which may be either deliberately applied or otherwise. Mutant strains may be produced by a variety of methods including ionising radiation, chemical treatment and genetic techniques, such as those outlined in Techniques for the Development of Micro-Organisms by H. I. Adler in "Radiation and Radio-isotopes for Industrial Microorganisms", Proceedings of the Symposium, Vienna, 1973, p. 241, International Atomic Energy Authority.

In the preparation of NCIB 11261, we used γ-radiation, e.g. of about 80 kilorads, NCIB 11261 has been found to show a requirement of uracil for growth, and the yield of the compounds of the invention has been found to be dependent to some extent on the amount of uracil present in the fermentation medium. It is preferred that the level of uracil is not greater than 200 μg/ml of broth, and preferably from 5 to 125 μg/ml.

The production of a compound of formula (I) wherein R is hydroxymethyl, formyloxymethyl or carboxyl or a salt of a compound in which R is carboxyl by fermentation of *Streptomyces clavuligerus* may be effected by conventional means, i.e. by culturing the *Streptomyces clavuligerus* in the presence of assimilable sources of carbon, nitrogen and mineral salts. Where a compound of formula I in which R represents an esterified carboxyl group is desired the corresponding compound in which R is carboxyl may be esterified either after isolation of the clavam carboxylic acid or *in situ* followed by isolation of the desired ester. Cultivation will preferably be carried out by submerged culture under aerobic conditions.

Assimilable sources of carbon, nitrogen and minerals may be provided by either simple or complex nutrients. Sources of carbon will generally include glucose, starch glycerol, molasses, dextrin, lactose, sucrose, carboxylic acids, alcohols, for example, methanol, *n*-paraffins and vegetable oils.

Sources of nitrogen will generally include soyabean meal, corn steep liquors, distillers solubles, yeast extracts, cottonseed meal, peptones, casein, amino acid mixtures, ammonia (gas or solution), ammonium salts or nitrates. Urea and other amides may also be used.

Nutrient mineral salts which may be incorporated into the culture medium include the generally used salts capable of yielding sodium, potassium, ammonium, iron, magnesium, zinc, nickel, cobalt, manganese, vanadium, chromium, calcium, phosphate, sulphate, chloride and carbonate ions.

An antifoam may be present to control excessive foaming and added at intervals as required.

Cultivation of the *Streptomyces clavuligerus* will generally be effected at a temperature of from 20°—32°C preferably of from 25—30°C, and will desirably take place with aeration and agitation, e.g. by shaking or stirring. The growth medium may initially be inoculated with a small quantity of sporulated suspension of the microorganism but in order to avoid a growth lag a vegetative inoculum of the organism may be prepared by inoculating a small quantity of culture medium with the spore form of the organism, and the vegitative inoculum obtained may be transferred to the fermentation medium, or, more preferaby to one or more seed stages where further growth takes place before transfer to the principal fermentation medium.

In a preferred embodiment of the fermentation a slope of *Streptomyces clavuligerus* may be used to inoculate a medium comprising sources of assimilable carbon, e.g. sucrose and/or glycerol, assimilable nitrogen, e.g. tryptones and/or complex mixtures of assimilable carbon and nitrogen, e.g. distiller's solubles and/or

yeast extracts, and nutrient minerals. Where a compound of formula I in which R is a hydroxymethyl or formyloxymethyl group is desired a slope of *Streptomyces clavuligerus* NCIB 11261 is preferred but a slope of *Streptomyces clavuligerus* NCIB 11260 is preferred where a compound of formula I in which R is a carboxyl group or an ester or salt thereof is desired. In the case of strain NCIB 11261, addition of uracil may be necessary. This medium may be allowed to grow for up to 3 days at from 25—30°C with agitation.

The inoculum thus formed may then be used to inoculate (in a quantity of up to about 10%) a nutrient medium containing simple or complex sources of assimilable carbon and nitrogen, and minerals, and in the case of strain NCIB 11261, uracil. Growth will desirably be carried out at from 25—30°C with agitation and aeration, in one or more stages. The final fermentation stage is normally effected in 2 to 10 days.

The compounds of the invention may be isolated from the fermentation medium by conventional isolation techniques. In order to minimise degradation of the compounds in solution, the pH during isolation is preferably maintained between 5 and 7. Thus, in general, the fermentation broth will be subjected to filtration, centrifugation and/or other techniques which will remove solid material and provide a clear solution containing the three fermentation-derived compounds of the invention namely the compounds of formula I wherein R is hydroxymethyl, formyloxymethyl and carboxyl and/or a salt thereof. It may also be possible to use the unclarified broth in certain adsorption-elution stages. The compounds may then be isolated by a variety of fractionation techniques, for example adsorption-elution, precipitation, fractional crystallisation and solvent extraction, which serve to remove other constituents of the fermentation broth and to separate the compounds of the invention from each other.

Thus, for example, the fermentation broth from which solid material has been removed may be applied to one or more materials which may retain either the desired compound or the undesired contaminants.

Thus, for example, the broth may be treated with an adsorbent carbon on which all three fermentation-derived compounds of the invention are adsorbed. This assists in separating unwanted broth components, particularly unwanted salts, from the desired compound. In general, the clarified broth may be passed through a carbon bed, e.g. in a column, preferably using just sufficient carbon to adsorb all the desired compound usually in a ratio of about 1 part by volume of carbon to 3—10 parts by volume of clarified broth.

The desired substance may then be eluted from the carbon with an aqueous water-miscible organic solvent, e.g. an alcohol, such as ethanol or isopropanol, or a ketone such as methyl ethyl ketone, methyl isobutyl ketone or, preferably, acetone, advantageously at a concentration of from 30% to 95% ketone, preferably 50 to 70%. Before elution, the carbon is preferably washed, e.g. with water, to remove non-adsorbed broth components.

In another procedure in which the hydroxymethyl and formyloxymethyl compounds of the invention are retained on adsorbent material, the unclarified or clarified broth may be passed through a suitable resin, e.g. a non-ionic resin such as the polystyrene resin XAD-4 (Surface Area 750 m^2/gm; Average Pore Diameter 50 Å; Porosity 0.50 to 0.55 ml pore/ml bead; sold by Rohm & Haas (UK) Ltd. Croydon, England). The resin will desirably be washed, e.g. the water, to remove impurities without eluting the desired compounds and the desired compounds may then be eluted. In the case of XAD-4 resin, a suitable eluant is an aqueous solution of an alkanol, e.g. methanol, or a ketone, e.g. acetone.

Alternatively, the clarified or unclarified fermentation broth or other solution containing one or more of the compounds of the invention in an aqueous medium, which may contain a water-miscible solvent, may be applied to an adsorbent, e.g. in a column, which does not retain the particular desired compound of the invention but which retains a significant quantity of other material, which may include unwanted compounds of the invention. In one embodiment of this procedure, an aqueous solution containing the desired hydroxymethyl and/or formyloxymethyl compounds, e.g. the clarified or unclarified broth or the solution in an aqueous water-miscible solvent (e.g. acetone) obtained as eluate from adsorbent carbon as described above, may be passed through a column of an anion-exchange resin. Where present, the acid according to the invention and other acids (such as clavulanic acid) will be retained while the formyloxymethyl and hydroxymethyl compounds of the invention are not retained. The resulting solution may then be subjected, if

required, to further fractionation. As described in detail hereinafter, the acid of the invention or a salt thereof may be subsequently eluted from the resin.

The anion exchange resin will generally carry amino groups (weakly basic) or quaternary ammonium groups (strongly basic). The resin may, for example, be a polystyrene, polyacrylic, epoxy-polyamine, phenolic-polyamine or cross-linked dextran resin and may be macroreticular or microreticular. The term 'resin' is used herein for convenience also to include cellulosic derivatives and the above dextran derivatives which are derived from naturally occurring polymers. Typical weakly basic ion exchange resins include Amberlite IRA 68 (Microreticular: polyacrylate: tertiary amino groups), and Amberlite IRA 93 (Macroreticular: polystyrene cross-linked with divinylbenzene: tertiary amino groups) both sold by Rohm & Haas (U.K.) Ltd. of Croydon, England. (Amberlite is a registered Trade Mark). Typical strongly basic ion exchange resins include Zerolit FF, Zerolit FF (ip) (Sold by Zerolit Co. Ltd.) and AG1-X2 (Bio-Rad Laboratories, Richmond, California). (Zerolit is a registered Trade Mark).

In another embodiment, the clarified or unclarified broth or other solution containing at least the hydroxymethyl and/or formyloxymethyl compounds of the invention may be applied to a non-ionic resin, e.g. the polystyrene resin XAD-2 (Surface Area 330 m^2/gm; Average Pore Diameter 90 Å; Porosity 0.40—0.45 ml pore/ml bead; sold by Rohm & Haas (U.K.) Ltd.) which does not retain the hydroxymethyl and formyloxymethyl compounds of the invention but which will retain several other significant broth components.

In a still further alternative, the hydroxymethyl and/or formyloxymethyl compounds of the invention may be extracted into a water-immiscible solvent, e.g. an ester solvent such as ethyl acetate or an alcohol such as butanol. Such extraction may be applied to the clarified or unclarified broth, or to the eluates or effluents from the foregoing adsorption-elution procedures, if necessary after removal of any water-miscible organic solvents which may be present. If the extraction is effected in the pH range 5—8, the acid according to the invention and similar acids, where present, will be in salt form and will remain in the aqueous phase. As indicated hereinafter, solvent treatment at acid pH enables the acid of the invention to be extracted.

To permit extraction into a suitably small volume of water-immiscible solvent it may be desirable to concentrate the solution, e.g. by evaporation under reduced pressure. A high concentration of a salt such as ammonium sulphate assists the extraction.

The desired compound of formula (I) wherein R represents a hydroxymethyl or formyloxymethyl group may be further purified by chromatography, e.g on silica or an organic solvent-compatible, cross-linked dextran such as Sephadex LH 20 (sold by Pharmacia U.K. Ltd.). (Sephadex is a registered Trade Mark). The solution of the compound obtained from the previous purification stage may be too dilute for application to the column and may conveniently be concentrated by evaporation under reduced pressure.

The column carrying the desired compound may then be eluted, for example using a solvent of suitable polarity. In the case of silica columns, ethyl acetate containing a hydrocarbon, e.g. hexane or toluene, may be used to elute the formyloxymethyl compound and the same solvent mixture or ethyl acetate alone may be used to elute the hydroxymethyl compound. In the case of Sephadex LH20, a suitable eluant is ethyl acetate alone. In general, the first fractions eluted contain predominantly the compound of formula I in which R is a formyloxymethyl group and the compound of formula I in which R is a hydroxymethyl group is eluted in the later fractions.

Finally, the fractions containing the desired compound may be combined and evaporated to yield the desired compound.

By a suitable combination of the foregoing procedures, the compounds in which R is a formyloxymethyl or hydroxymethyl group have been isolated as pale yellow oils of at least 90% purity. However, in this form the compounds are unstable and are best stored in solution in water or organic solvents.

As indicated above, the acid of the invention can be separated from the hydroxymethyl and formyloxymethyl compounds and other broth components by application of the clarified or unclarified broth or other aqueous solution containing the acid and/or a salt thereof to an anion exchange resin. Such a resin is conveniently in the salt form, e.g. the chloride form. The acid of the invention, usually together with one or more other β-lactam carboxylic acids, notably clavulanic acid, may then be eluted from the ion-exchange resin, conveniently

using an aqueous solution of a salt as eluant. We have found that solutions of lithium salts, e.g. at a molarity 0.2 to 2.5M, are particularly useful as eluants since the lithium salt of one of the principal contaminating acids, clavulanic acid, can be removed by fractional crystallisation to leave the lithium salt of the acid of formula I in the mother liquor. In general, the eluate containing excess lithium salt eluant, will be concentrated, advantageously to a molarity of lithium salt of about 5—10, before precipitation of the lithium clavulanate. If desired, however, the lithium clavulanate can be salted out by addition of still further lithium salt. Alternatively, the acid of the invention may be eluted with an aqueous eluant, followed by addition of lithium salt to the eluate.

However, in order to minimise elution of adsorbed impurities from the resin, it may be advantageous to include in the eluant a water-miscible organic solvent at high concentration. Alternatively, after elution in the absence of such a solvent, this can be added to the eluate to precipitate eluted impurities and the precipitate separated off before further treatment. The solvent may, for example, be a ketone such as acetone, an alcohol such as methanol, ethanol, isopropanol or ethylene glycol, an ether such as dioxan or tetrahydrofuran or a substitued amide, imide or sulphoxide solvent such as dimethylformamide or dimethylsulphoxide. In general, alcohols are preferred as such solvents, e.g. ethanol or isopropanol, the preferred concentration thereof in the eluant or eluate after addition of the alcohol thereto being from 70 to 97% by volume. Undesirable material which is precipitated may be separated, e.g. by centrifugation or filtration. Fractional crystallisation as described above, to remove an undesired major component of the supernatant, or filtrate, yields a mother liquor from which the acid of the invention or a salt thereof may be isolated.

When the clarified or unclarified broth is applied directly to the anion exchange resin, the salts therein tend to overload the resin. It is often preferable, therefore, to remove salts by a preliminary adsorption of the active materials onto adsorbent carbon, followed by elution with an aqueous water-miscible solvent.

The acid according to the invention or a derivative thereof may be isolated from the mother liquor remaining after removal of the clavulanate by any convenient means, but the method employed will, of course, depend on whether or not it is the acid of the invention or a salt or ester thereof that it is desired to isolate. Further purification means will be desirable in order substantially to free the compound of the invention from minor amounts of impurities which may have been carried through the earlier purification steps.

The acid of the invention may, for example, be separated from the mother liquor remaining after removal of the clavulanate by solvent extraction at acidic pH, preferably at a pH less than 3.0, using a water-immiscible solvent, for example an ester such as ethyl acetate, a ketone such as methyl isobutyl ketone or an alcohol such as butanol.

Alternatively, ion-pair extraction can be used, for example by extracting the aqueous medium containing the desired acid and/or salt thereof with a solution of an amine in a water-immiscible solvent (e.g. a hydrocarbon such as hexane or kerosene or a halogenated hydrocarbon such as methylene chloride). The amines are preferably water insoluble, for example, carrying one or more long-chain aliphatic groups, e.g. a branched chain primary or secondary alkylamine in the molecular weight range 280—400g for example, Amberlite XLA3, LA1 and LA2 and Primene JMT (Sold by Rohm & Haas (U.K. Ltd.). (Primene is a registered Trade Mark). The pH of the system should be acidic (e.g. in the range 3—7) so that the amine is in the salt form and the acid of the invention in the acid form. The desired acid is extracted into the solvent phase to form a salt with the amine. The acid pH of the system may be achieved by addition of a mineral acid such as hydrochloric acid or an organic acid such as acetic acid.

If desired, the acid may be back-extracted from the organic solvent solution into an aqueous medium containing alkali. If an amine is present in the solvent phase, the aqueous medium may be an aqueous salt solution.

Where an ester is desired, an extract of the acid may be treated with an esterifying agent, e.g. a diazoalkane or diazoaralkane, for example diazomethane or diphenyldiazomethane. The resulting solution, which contains an ester compound of the invention in an organic solvent, may be too dilute for application to a column for purification purposes and will thus, if this mode of purification is desired, preferably be evaporated to dryness followed by redissolution into a smaller volume of solvent. A desirable column material is silica.

The column carrying the desired ester compound may then be eluted, for

example using a solvent of suitable polarity, e.g. in the case of silica columns, ethyl acetate containing a hydrocarbon such as hexane or toluene.

Finally, the fractions containing the desired ester compound may be combined and the desired compound obtained by crystallisation. By the foregoing procedures, the diphenylmethyl ester of the invention has been isolated as white needle crystals of high purity.

Alternatively, where a carboxylic acid of the invention is prepared and it is desired to isolate a salt of the invention, the mother liquor may be extracted as described above, and this extract may then be treated with a suitable base to form a salt according to the invention.

The base may be an organic solvent-soluble base such as sodium 2-ethylhexanoate which will give a salt product. Alternatively, the extract may be treated with an aqueous solution of a water-soluble base to form an aqueous solution of the desired salt. In the latter case, this may be further purified by chromatography, for example, on an anion-exchange resin in the salt, e.g. chloride, form. A suitable resin is AG1-X2 (Bio-Rad Laboratories; Richmond, California). The acid of the invention is retained on this resin and may be eluted by a salt gradient, the anion of which desirably being the same as that already present on the column, and the cation of which being that of which it is desired to prepare a salt of the invention. Thus, for example, the sodium salt of the desired acid can be eluted with aqueous sodium chloride at gradually increasing concentration, e.g. from 0.1M to 0.25M.

Eluted fractions containing the desired compound in salt form together with excess eluant salt will desirably be combined and applied to a column which will retain the organic material in the presence of excess salt but allow passage therethrough of the excess salt. A suitable material for this purpose is adsorbent carbon. Elution of this column may be effected with an aqueous water-miscible solvent, e.g. a ketone such as methyl ethyl ketone, or, preferably, acetone. Fractions may be collected and combined and a salt of the invention obtained by removal of the solvent, e.g. by evaporation or lyophilisation.

Where the acid of formula I or a salt thereof has been isolated but an ester thereof is required, the acid or salt may be subjected to esterification. Similarly, where a particular ester has been isolated but another ester is required, the initial ester may be deesterified and the acid then reesterified. In this latter case, the initial ester-forming group will preferably be readily removable such as an arylmethyl group.

Such deesterification may conveniently be carried out by reductive deesterification techniques using esters susceptible to reductive cleavage, e.g. arylmethyl esters, such as benzyl, benzhydryl, trityl or *p*-nitrobenzyl esters. Such reductive deesterification may be carried out by, for example, hydrogenolyis, e.g. using a metal catalyst, for example, a noble metal such as platinum, palladium or rhodium. The catalyst may be supported, e.g. on charcoal or kieselguhr. In the case of *p*-nitrobenzyl esters, cleavage may be effected by reduction of the nitro group (e.g. using a dissolving metal reducing agent such as zinc in acetic acid or zinc in aqueous tetrahydrofuran or acetone controlled in the pH range 3—6, preferably 4—5.5, by the addition of aqueous HCl; aluminium amalgam in a moist ether e.g. tetrahydrofuran; or iron and ammonium chloride in an aqueous ether such as aqueous tetrahydrofuram) followed by hydrolysis either under the reduction conditions or by subsequent treatment with acid.

The alkyl, alkoxyalkyl and aralkyl esters may be prepared by reaction of the acid of formula I with the appropriate diazoalkane or diazo-aralkane, e.g. diazomethane or diphenyldiazomethane. The reaction will conveniently be effected in an ether, alcohol, ketone, ester or halo-hydrocarbon solvent, e.g. diethyl ether, butanol, methyl-isobutyl ketone, ethyl acetate or dichloromethane.

The esters derived from alcohols may be produced by reaction of a reactive derivative of the alcohol, for example, a halide such as the chloride, bromide or iodide, or a hydrocarbon-sulphonyl derivative such as a mesyl or tosyl ester, with a salt of the acid of formula I, e.g. an alkali or alkaline earth metal salt such as a lithium, sodium, potassium, magnesium or calcium salt, or an amine salt such as triethylammonium salt. This reaction is preferably carried out in a sulphoxide or amide solvent, e.g. dimethyl sulphoxide, dimethylformamide or hexamethylphosphoramide.

Compounds of the invention and methods for their preparation and isolation will now be described in the following non-limiting Examples.

In the Examples which follow the following steam-sterilised media were used:

In relation to infrared spectra the symbols s, m and w refer to strong, medium and weak intensity respectively.

Medium A
Soya bean meal 5 g/l, yeast extract 5 g/l, tryptone 5 g/l, K_2HPO_4 0.2 g/l, glycerol 10 g/l and tap water to 1 litre.

Medium B
Glycerol 35 g/l, citric acid 1.5 g/l, L. asparagine 6.7 g/l, $MgSO_4 . 7H_2O$ 0.5 g/l, K_2HPO_4, 0.21 g/l, KH_2PO_4 0.42 g/l, $CaCl_2$ 0.2 g/l, $ZnSO_4 . 7H_2O$ 0.05 g/l, $FeSO_4 . 7H_2O$ 0.03 g/l, $MnSO_4 . 4H_2O$ 0.1 g/l and distilled water to 1 litre.

Medium C
Sucrose 20 g/l, distillers solubles 15 g/l, yeast extract 0.2 g/l, K_2HPO_4 0.2 g/l, tryptone 5 g/l, glycerol 10 g/l and tap water to 1 litre.

Medium D
Soya meal (unmilled) 30 g/l, ferric sulphate 0.1 g/l, KH_2PO_4 0.1 g/l, soluble starch 47 g/l, silicone antifoam emulsion 0.05 (% v/v) and tap water to 1 litre.

The silica used in column chromatography procedures is Woelm silica (activity grade III).

Example 1
2-Hydroxymethyl-clavam

a) Fermentation of *Streptomyces clavuligerus* strain

Strepomyces clavuligerus strain NCIB 11261 was maintained on malt agar slopes (malt extract 24 g/l; yeast extract 5 g/l; agar 15 g/l; adjusted to pH 7.8) grown for 2 weeks at 28°C.

The slopes were developed for shake flask fermentation with 1/3 of a slope being used to inoculate 50 ml of Medium A (pH adjusted to 6.5) in a 250 ml flask.

This was incubated at 28°C for 42 h at 220 rev/min on a rotary shaker with a 2" throw. 2 ml of the inoculum was used to inoculate 50 ml of Medium B containing NaCl (0.1 g/l) and uracil (0.01 g/l) (pH adjusted to 7.0 with KOH) in a 250 ml. unbaffled flask. This was kept at 28°C for 72 h on a rotary shaker with a 5 cm throw at 220 rev/min.

b) Isolation of 2-hydroxymethyl-clavam

Broth (5l) prepared in a) above was centrifuged and the supernatant applied to a column containing XAD-2 resin (bed ht. 100 cm: diam 2.5 cm). The effluent was saturated with ammonium sulphate and extracted with ethyl acetate (3×1 litre). The extracts were combined, dried over sodium sulphate and evaporated to dryness under reduced pressure.

The residue was dissolved in a little ethyl acetate, silica added and the mixture evaporated under reduced pressure. The resultant solid was applied to the top of a column containing dry silica (30×1 cm) and the column eluted with toluene-ethyl acetate (1:1), 20 ml fractions being collected. Fractions 11—15 were combined and evaporated under reduced pressure to give the alcohol as a yellow oil (40 mg).

The infrared spectrum of a bromoform solution of the alcohol showed peaks (cm^{-1}) at 3660 w, 3560 m, 2930 n, 1770 s, 1600 w, 1455 w, 1392 m, 1332 s, 1233 m, 1036 s, 983 m, 948 m, 910 w, 848 m and 822 w. The 100 MHz proton nmr spectrum of a solution in deuteroacetone is shown in Table 1. A field desorption mass spectrum gave a peak at m/e 143, corresponding to a molecular weight of 143.

The infrared and nmr spectra are shown in Figs. 2 and 1 respectively.

Example 2
2-Hydroxy-methyl-clavam

Broth (2l) prepared as in Example 1(a) above was centrifuged and the supernatant applied to a column containing XAD-4 resin (bed height 50 cm; diameter 2.5 cm). The resin was washed with water, then eluted with methanol-water (1:1 by vol.). The eluate was evaporated under reduced pressure to an aqueous solution.

The aqueous solution (50 ml) was extracted with 4×50 ml ethyl acetate and the extracts were combined, dried over Na_2SO_4 and evaporated under reduced pressure to a residue.

The residue was dissolved in a little ethyl acetate, silica was added and the

mixture evaporated under reduced pressure. The resultant dry silica was applied to a column containing dry silica (Woelm, act. grade III, 20×1 cm). Elution was with toluene-ethyl acetate (1:1) and 20 ml fractions were collected. Fractions 10—12 were combined and evaporated to give 13 mg of a pale-yellow oil.

The product had the following properties

TLC. Portions of the product were subjected to TLC on silica with the solvents given below. Assays were by overlay with *Saccharomyces carlsbergensis*.

Solvent	Support	Rf
Methanol	EK6061 (0.1mm thick plastic backed)	0.79
Ethyl acetate	EK6061	0.62
	Merck 60 (plastic backed)	0.40
Chloroform	EK6061	0.26
	Merck 60	0.04
Toluene	EK6061	0
	Merck 60*	0
Toluene-ethyl acetate (1:1)	EK6061	0.41
	Merck 60	0.18
Toluene-methanol (9:1)	EK6061	0.40
	Merck 60	0.15

(E. Merck is a registered Trade Mark)

UV Spectrum

Addition of 2 drops of 2M NaOH to a methanolic solution resulted in a strong absorption with λ_{max} 270 nm.

The infrared and nmr spectra were similar to those of the product of Example 1.

Example 3
2-Hydroxymethyl-clavam

a) *Inoculum* development

Streptomyces clavuligerus NCIB 11260 was stored freeze-dried in ampoules. The contents of one ampoule was suspended in sterile distilled water and then added to Medium C adjusted to pH 6.5 with hydrochloric acid in a 250 ml shake flask. The flask was incubated on a rotary shaker at 220 rev/min with a 2″ throw at 26°C for 72 h.

A portion (2 ml) of the 72 h inoculum was used to inoculate each of 4×2 litre florence flasks containing 150 ml of the above medium. The florence flasks were incubated on a rotary shaker at 220 rev/min with a 2″ throw at 26°C for 48 h.

The contents of three of the florence flasks (3×150 ml 3.75 v/v) were used to inoculate 3×5 litre fermenters each containng 4 litres of Medium D (adjusted to pH 6.5 with NaOH/HCl).

These fermenters were agitated with 2×3″ diameter 4 bladed impellers at 750 rev/min at 28°C for 20 h with an air flow of 3 litres/min.

Inoculum (7.5 litres, 5% v/v) from the 5 litre fermenters are used to inoculate 150 litres of the above soya meal medium in a 230 litre stainless steel fermenter. The vessel was agitated with a six bladed 8″ impeller at 350 rev/min and aerated at 420 litre/min for 20 h at 28°C.

b) Fermenation

Broth from the 230 litre fermenter (50 litres, 10% v/v) was used to inoculate 430 litres of Medium D in a 700 litre stainless steel fermenter. The vessel was agitated with a six bladed 10″ impeller at 350 rev/min and aerated at 280 litre/min. The fermentation was carried out at 28°C for 92 h, and was maintained at pH 6.5.

c) Extraction of fermentation broth

Harvest broth (530 l, pH 6.5) from step (b) was adjusted to pH 5.3 with sulphuric acid, filter aid (15 kg) added, and the mixture clarified on a rotary drum filter with a cellulose precoat. The filtrate was adsorbed onto carbon (Pittsburgh CAL, 105 l) in columns. The carbon was washed with water (70 l) and the columns were eluted with aqueous acetone (60% v/v, 135 l).

The combined eluates (148 l) were applied to a column of IRA68 resin

(chloride cycle, 12.4 l), the effluent being collected. The column was washed with water (20 l).

d) Isolation of 2-hydroxymethyl-clavam

The combined effluent and washings (148 l) from step (c) were concentrated in a pot still to 50 l, and the pH adjusted to 7.0 with 40% aqueous sodium hydroxide solution. A portion (25 l) of this concentrate was saturated with ammonium sulphate and stirred with ethyl acetate (25 l) for 30 min. After standing for 1 h the organic phase was separated, dried with anhydrous magnesium sulphate and concentrated under reduced pressure to yield the *title compound* (46 g) as an oil.

A portion of this oil (13.6 g) was extracted with ethyl acetate (25 ml), insoluble material filtered off and washed with a further 5 ml ethyl acetate. Combined filtrate and washings were applied to a column (bed ht. 53 cm, diam. 5.2 cm) of Sephadex LH20 packed in ethyl acetate. The column was eluted with ethyl acetate, 50 ml fractions being collected. Fractions 36—42 were combined and evaporated to yield a yellow oil (1.17 g; λ_{max} 261 nm $E^{1\%}_{1cm}$ 273, M/10 NaoH).

A portion of this oil (960 mg) was further purified by dissolving in toluene-ethyl acetate (1:1; 2 ml) and applying to a dry column (bed ht. 23 cm, diam. 2.1 cm) of silica powder. The column was developed and eluted with toluene-ethyl acetate (1:1), the eluate being collected in 10 ml fractions. After 380 ml eluate had been collected the eluting solvent was changed to ethyl acetate. Fractions 45—73 were combined and evaporated to give the *title compound* as a pale yellow oil (220 mg).

The i.r. and n.m.r. spectra of the product were substantially the same as those obtained for the product of Example 1. The maximum $E^{1\%}_{1cm}$ value reached by a solution of the compound in M/10 NaOH was 841 at λ_{max} 259 nm. $[\alpha]_D^{23}$ in dimethyl sulphoxide −166°.

Example 4
2-Formyloxymethyl-clavam

a) Fermentation of *Streptomyces clavuligerus* strain

Streptomyces clavuligerus strain NCIB 11261 was maintained on malt agar slopes (malt extract 24g/l; yeast extract 5g/l;, agar 15g/l; adjusted to pH 7.8) grown for 2 weeks at 28°C. Sterile water (8 ml) was added to each slope and a suspension made. 2ml portions of this suspension were used to inoculate each of four 250 ml baffled shake flasks containing 50 ml of Medium A containing uracil (100 μg/ml) (pH adjusted to 6.5 with HCl).

The flasks were incubated on a rotary shaker at 220 rev./min with a 5 cm throw, at 26°C for 24 h. The shake flask contents were bulked and 0.5 ml portions of this inoculum used to inoculate 32×250 ml baffled shake flasks containing the above medium and incubated for 48 h under the previous conditions. 120 ml (3% v/v) portions of this bulked 48 h inoculum were used to inoculate 6×5 litre fermenters each containing 4 litres of Medium B (pH adjusted to 7.0 with NaOH) additionally containing varying levels of uracil.

The uracil concentration and the aeration rate in each fermenter is set out below:

5 litre Fermenter No.	Uracil concentration μg/ml	Aeration rate litre/min.
1	100	1.5
2	100	3
3	100	6
4	50	6
5	35	6
6	25	6

The 5 litre fermenters were agitated at 250 rev./min with 2×3½″ diameter 4 bladed impellers. The fermentations were maintained at 28°C for 94 h.

b) Isolation of 2-formyloxymethyl-clavam

Bulked broth from fermenters 1—6 above (18 l) was centrifuged and the supernatant applied to a column containing XAD-4 resin (bed ht. 130 cm; diam. 2.8 cm). The resin was washed with water (4 l) and eluted with acetone-water (4:1) (c. 500 ml). The eluate was evaporated to 100 ml under reduced pressure, the pH adjusted to 6.8 with sodium hydroxide, the solution saturated with ammonium sulphate and extracted with ethyl acetate (4×100 ml). The combined extracts were dried (sodium sulphate), evaporated under reduced pressure and the residue

dissolved in a little ethyl acetate. Silica was added and the mixture evaporated under reduced pressure to give a solid which was added to the top of a column containing dry Woelm silica (act. grade III; bed ht. 50 cm; diam. 2.9 cm). The column was eluted with toluene-ethyl acetate (1:1), 15 ml fractions being taken.

Fractions 4—11 were combined, evaporated under reduced pressure and the residue subjected to chromatography on silica (bed ht. 30 cm; diam. 2.1 cm), with hexane-ethyl acetate (2:1) as eluant, 10 ml fractions being collected. Fractions 17—23 were combined and evaporated under reduced pressure to give the formyloxy compound as a pale yellow oil (137 mg). The infrared spectrum of a bromoform solution of the sample is shown in Fig. 3 of the accompanying drawings and has peaks (cm^{-1}) at 2915 m, 1784 s, 1730 s, 1462 w, 1406 w, 1392 w, 1346 m, 1282 m, 1066 s, 1034 s, 990 m, 940 w, 926 w and 863 w. A 100 MHz proton nmr spectrum of a solution in deuterochloroform had τ values as shown in Table 2.

Broth (50 μl) from 5 litre fermenter No: 6 was applied to Merck silica 60 F254 plates, glass-backed, and the plates developed with ethyl acetate, air-dried and overlayed with nutrient agar containing *Saccharomyces carlsbergensis* 1738. The formyloxy compound was found to have an R_f value of 0.89. The formyloxy compound was also detected by quenching of fluorescence under u.v. light (254 nm) after treatment of the developed TLC plate with ammonia vapour.

Example 5
2-Formyloxymethyl-clavam

Filtered broth (25 μl) obtained as in Example 3 was applied to Merck silica 60 F254 plates, glass-backed, which were developed with ethyl acetate and air-dried. The plates were then saturated with ammonia vapour and the chromophore produced on reaction with ammonia detected under u.v. light (254 nm). The formyloxy compound was found to have an R_f value of 0.85.

Example 6
2-Formyloxymethyl-clavam

a) Inoculum development

Streptomyces clavuligerus strain NCIB 11261 was maintained on malt agar slopes (malt extract 2.4%; yeast extract 0.5%; agar 1.5% w/v; pH 7.8) grown for 2 weeks at 28°C. Tween 80 solution (8 ml) (Tween is a registered Trade Mark) was added to the slope and a suspension made. 2 ml portions of this suspension were used to inoculate each of four 250 ml baffled shake flasks containing 50 ml of Medium A containing uracil (100 μg/ml) (pH adjusted to 6.8—7.1).

The flasks were incubated on a rotary shaker at 220 rev./min with a 2″ throw, at 28°C for 48h. 2 ml portions of this inoculum were used to inoculate identical shake flasks containing the above medium and incubated for 24 h. under the previous conditions. The shake flask contents were bulked and 120 ml portions transferred to 250 ml aspirators. These 120 ml (3% v/v) portions were then used to inoculate 5 litre fermenters each containing 4L of Medium B containing NaCl (0.1 g/l), antifoam (0.05% v/v) and uracil (35 μg/ml), (pH adjusted to 7.0 with KOH).

The fermenters were agitated at 250 rev./min with 2×3½″ diameter 4 bladed impellers. The fermentations were maintained at 28°C for 24 h. at an aeration rate of 6 l/min.

7.5 l (5% v/v) of this 24 h culture were used to inoculate a 230 litre fermenter containing 150 l. of the above medium. The fermentation was maintained at 28°C for 93 h. at an aeration rate of 5 cu. ft/min and an agitation rate of 250 rev/min.

Further antifoam was added as required throughout the fermentation.

b) Isolation of 2-formyloxymethyl-clavam

(i) Harvest broth (133 l) was adjusted to pH 7.0 with sodium hydroxide and clarified by centrifugation. To the clear supernatant (114 l) 1/3 volume butanol was added, stirred for 30 mins and the phases separated by centrifugation. The aqueous phase (118 l) was re-extracted, separated with butanol as before, and the bulked butanol extracts (76 l) washed with distilled water (40 l).

Distilled water (40 l) was added to the butanol extracts and the azeotrope concentrated to 1 l by means of a pot still and evaporation under reduced pressure. The concentrate was saturated with ammonium sulphate and extracted with 4×¼ volume ethyl acetate. The bulked ethyl acetate extracts (1.95 l) were dried with magnesium sulphate, evaporated to 500 ml, dry silica added (Sorbsil M60, 50 g) and evaporated to dryness.

The solid residue was added to the top of a column containing dry silica (Sorbsil M60, 142x3.8 cm) (Serbsil is a registered Trade Mark) and the silica was eluted with toluene/ethyl acetate (1:1), 25 ml fractions being collected. Fractions 38—73 inclusive were bulked.

(ii) Harvest broth (133 l) prepared as in (a) above was clarified by centrifugation and the supernatent (123 l) evaporated to 221 on a pot still. The concentrate was adjusted to pH 7.0 with sodium hydroxide, filtered, saturated with ammonium sulphate and extracted with 2x½ volume ethyl acetate. The bulked ethyl acetate extracts (21.5 l) were washed with saturated ammonium sulphate solution (5 l), dried with magnesium sulphate and evaporated to 250 ml under reduced pressure. Sorbsil silica (50 g) was added, the mixture evaporated to dryness and the solid residue added to the top of a column containing dry silica (Sorbsil M60, 138x3.8 cm). The silica was eluted with toluene/ethyl acetate (1:1), 25 ml fractions being taken. Fractions 42 to 60 were bulked.

(iii) The bulked eluates from (i) and (ii) were combined, evaporated to 15 ml and applied to a column containing LH20 Sephadex (60x60 cm) in ethyl acetate. The Sephadex was eluted with ethyl acetate, 25 ml fractions being collected. Fractions 38—46 were bulked and evaporated to dryness to yield *title compound* (700 mg) having characteristics similar to those described in Example 4.

Example 7

Methyl clavam-3-carboxylate

a) Extraction of fermentation broth

Harvest broth (520 l, pH 6.7) obtained as in Example 3 was adjusted to pH 5.4 with sulphuric acid, filter aid (15 Kg) added, and the mixture clarified on a rotary drum filter with a precoat. The filtrate was adsorbed on to carbon (Pittsburgh CAL, 105 l) in columns. the carbon was washed with water (70 l) and the columns were eluted with aqueous acetone (60% v/v, 135 l).

The combined eluates (143 l) were applied to a column of IRA 68 resin (chloride cycle, 12.5 l). The column was washed with water (20 l) and eluted with 5% (w/v) aqueous lithium chloride solution (16 l), collecting the eluate in fractions of 1 l. Fractions 6—15 were combined and cold propan-2-ol (5 vol.) was added with stirring, followed by filter aid (500 g) and the mixture filtered. The cake was washed with water/propan-2-ol (1:5, 2—4 l) and the combined filtrates concentrated under reduced pressure to 1/6 volume of combined eluates. The resulting concentrate was stored at 4°C for 16 h, during which time a solid formed. The solid was removed by filtration and washed with 30% aqueous lithium chloride solution (3x50 ml).

b) Isolation of methyl clavam-3-carboxylate

Mother liquor and washings from step (a) (2 l, pH 5.8) were saturated with about 800 g of ammonium sulphate, the pH adjusted to 3.0 with 11.4M hydrochloric acid and extracted with ethyl acetate (2x 500 ml). The ethyl acetate extracts containing the β-lactam acid were combined, dried over sodium sulphate and treated with excess diazomethane prepared by the method of Mlejnek (J. Chromatog. 1972, *70*, 59).

The ethyl acetate extract was evaporated under reduced pressure, the residue dissolved in a little ethyl acetate, silica added and the mixture evaporated under reduced pressure. The resultant solid was added to the top of a column containing dry silica (bed ht. 50 cm; diam. 2.9 cm) and the column eluted with hexane-ethyl acetate (1:1 by vol.), 20 ml fractions being collected. Fractions 8—14 were combined and evaporated under reduced pressure to yield a yellow oil (810 mg), which was dissolved in a little ethyl acetate, silica added and the mixture evaporated under reduced pressure. The resultant solid was added to the top of a column containing dry silica (bed ht. 40 cm; diam. 2.5 cm). The column was eluted with hexane-ethyl acetate (3:2 by vol.), 10 ml fractions being collected. Fractions 22—28 were combined and evaporated under reduced pressure to yield the methyl ester as a pale yellow oil (200 mg). Found: C, 49.1 49.3; H, 5.3, 5.3; N, 7.7, 8.1%; S, nil. $C_7H_9NO_4$ requires C, 49.1: H, 5.3; N, 8.2%. The infrared spectrum of a bromoform solution of the sample showed absorption peaks at 2950, 1785, 1754 and 1215 cm^{-1}. A 100 MHz proton n.m.r. spectrum of the ester in deuterochloroform solution had τ values of 4.52 (d, 2.5) (1H); 5.16 (dd, 4.5, 7.5) (1H): 5.88 (dd, 7.5, 11.5) and 6.90 (dd, 4.5 11.5) (2H); 6.24 (s) (3H); 6.69 (dd, 2.5, 16) and 7.14 (d, 16) (2H) and this is shown in Fig. 6.

Example 8

Isolation of diphenylmethyl clavam-3-carboxylate

Mother liquor and washings (2.7 l) obtained by the method described in Example 7 (a) were adjusted to pH 2.9 with hydrochloric acid and extracted with ethyl acetate (2×2 l). The combined ethyl acetate extract was dried over sodium sulphate and diphenyldiazomethane (40 ml, 0.5M solution in CH_2Cl_2) was added. The extract was evaporated under reduced pressure to 250 ml and a further 60—70 ml of the diphenyldiazomethane solution added until there was no evolution of gas and the deep red colour remained for about 15 min.

Silica was added to the ethyl acetate extract and the mixture was evaporated under reduced pressure. The resultant solid was added to the top of a column containing dry silica (bed ht. 107 cm, diam. 5 cm) and eluted with hexane-ethyl acetate (5:1 by vol.) 25 ml fractions being collected. Fractions 135—188 were combined and the diphenylmethyl ester, which crystallised during collection, was filtered off and washed with hexane to give white needles (40 mg). The supernatant was evaporated under reduced pressure and the residue recrystallised from toluene to give the *diphenylmethyl* ester as white needles (900 mg). M.P. 146.7°C (heating rate 2°/min); $[\alpha]_D^{23}$ −109° (0.1% w/v in dimethyl sulphoxide). Found C, 70.3; H, 5.3; N, 4.2; S, 0% $C_{19}H_{17}NO_4$ requires: C 70.6; H, 5.3; N, 4.3%. The infrared spectrum of a Nujol mull (Fig. 4) (Nujol is a registered Trade Mark) showed absorption peaks at 1780, 1750, 1200, 762 and 698 cm^{-1}; a solution in bromoform gave peaks at 1780, 1742 and 740 cm^{-1}. A 100 MHz proton n.m.r. spectrum of a deuterochloroform solution (Fig. 5) has τ values shown in Table 3. A field desorption mass spectrum showed a molecular ion peak at m/e 323.3 (expected m.w. for $C_{19}H_{17}NO_4$: 323.16).

The u.v. spectrum of a sample of the diphenylmethyl ester prepared in a manner similar to that described above and dissolved in methanol containing 1% v/v of 1M NaOH showed an $E_{1\,cm}^{1\%}$ value of 691 at $\lambda_{max}{}^{272nm}$ immediately after alkali treatment.

Example 9

Preparation of sodium clavam-3-carboxylate

Diphenylmethyl ester (80 mg) prepared as in Example 8 was dissolved in ethanol (30 ml) and stirred at room temperature for 30 min. under hydrogen (1 atmosphere) in the presence of 10% (w/w) palladium on charcoal (25 mg) and sodium bicarbonate (20 mg). The solution was filtered, water (100 ml) added and the mixture extracted with ethyl acetate (2×100 ml). The aqueous layer (pH 6.1) was freeze-dried to give the *sodium salt*, which showed characteristics similar to those of the product of Example 11.

Example 10

Preparation of lithium clavam-3-carboxylate

Diphenylmethyl ester (160 mg) prepared in Example 8, lithium carbonate (18.5 mg) and 10% (w/w) palladium on charcoal (50 mg) were stirred in ethanol (50 ml) under hydrogen (1 atmosphere) for 30 min. The solution was filtered, water (200 ml) added and the mixture extracted with ethyl acetate (2×100 ml). The aqueous layer (pH 4.8) was adjusted to pH 6.0 with lithium carbonate and freeze-dried to give the *lithium salt* (42 mg). The infrared spectrum of Nujol mull showed peaks at 3400, 1780 and 1600 cm^{-1}. A 100 MHz proton nmr spectrum of a heavy water solution had τ values of 4.48 (d,3) (1H); 5.24 (multiplet) (1H); 5.79 (dd, 8, 11) (1H) and 6.95 (dd, 5, 11) (1H); 6.56 (dd, 3, 17) (1H) and 7.08 (d,17) (1H).

Example 11

Isolation of sodium clavam-3-carboxylate

Mother liquors and washings (1 l) obtained by the method described in Example 7(a) were adjusted to pH 3.0 with concentrated hydrochloric acid and extracted with ethyl acetate (2×1 l). The bulked ethyl acetate extracts were back extracted with water (250 ml) at pH 6.5 (pH adjusted with 1M sodium hydroxide) and the resulting aqueous solution passed down a column containing AG1—X2 (Bio-Rad Laboratories) resin in the chloride cycle (column size: 74×2.5 cm). The column was washed with water (650 ml) and eluted with sodium chloride solution of gradually increasing concentration (500 ml, each of 0.1M, 0.15M, 0.2M, 0.25M), 25 ml fraction being taken.

Fractions 14 to 35 were bulked and passed down a column containing Pittsburgh carbon (column size: 16×2.5 cm). The charcoal was washed with two

bed volumes of water and eluted with acetone/water (4:1), 50 ml fractions being collected. Fractions 1 to 4 were bulked, the acetone removed by concentration under reduced pressure, and the residual solution freeze-dried to yield the *title salt* (1.4 g.).

The infrared spectrum of Nujol mull showed broad peaks at 3400, 1770 and 1600 cm^{-1}. A 100 MHz proton nmr spectrum of a heavy water solution had τ values of 4.48 (d,3) (1H); 5.26 (multiplet) (1H); 5.81 (dd, 8,11) (1H) and 6.96 (dd, 5,11) (1H); 6.56 (dd, 3,17) (1H) and 7.08 (d, 17) (1H).

The maximum $E^{1\%}_{1cm}$ value reached by a solution of the compound in 1M NaOH was 415 at λ_{max}258 nm.

Example 12
Magnesium clavam-2-carboxylate

Diphenylmethyl clavam-2-carboxylate (979 mg), a stoichiometric amount of $MgCO_3$ and palladium on charcoal (ca. 300 mg) were added to absolute ethanol (100 ml). The mixture was rapidly stirred in a hydrogen atmosphere until the uptake of hydrogen had fallen to such a rate as to indicate less than 1% reaction per minute. the mixture was filtered through Celite 535 (a diatomaceous earth supplied by Johns Manville Inc. U.S.A.) (Celite is a registered Trade Mark) and the Celite washed with water. The pH of the aqueous ethanol filtrate was adjusted to 6 with $Mg(OH)_2$ solution and the aqueous solution washed twice with ethyl acetate and freeze-dried to yield the *title salt* (31.5 mg), λ_{max}(0.1M NaOH) 259—260 nm, E^1_1 660.

Example 13
Methyl clavam-2-carboxylate

An aqueous ethanol solution of magnesium clavam-2-carboxylate was prepared from diphenylmethyl clavam-2-carboxylate (230 mg) in similar manner to that described in Example 12. The solution was adjusted to pH 2.0 with HCl solution and extracted twice with ethyl acetate. The bulked extracts were dried (Na_2SO_4), filtered and diazomethane gas passed through for ½ hr. The resulting solution was evaporated to yield the *title compound* (117 mg) as an oil, λ_{max}(EtOH+1% 1M NaOH) 272 nm, E^1_1 1080; $[\alpha]_D$ −124° (*c* 0.16; EtOAc).

Example 14
2-Hydroxymethyl-clavam (I) and 3-formyloxymethyl-clavam (II)

a) Harvest broth (130 l, pH 5.6) obtained as in Example 1 was clarified by centrifugation and the supernatant (110 l, pH 5.7) adsorbed onto carbon (Pittsburgh CAL, 13 l) in a column. The precipitate at the top of the column was removed, the carbon was washed with water (10 l) and the column was eluted with aqueous acetone (60% v/v, 40 l).

Combined eluates (78 l, pH 6.2) from two lots of harvest broth were applied to a column of IRA 68 resin (chloride cycle, 5 l), the effluent being collected and concentrated under reduced pressure. The pH of the concentrate (20 l) was adjusted to 7.0 with 40% aqueous NaOH solution. The concentrate was saturated with $(NH_4)_2SO_4$ and extracted with ethyl acetate (3×10 l). The bulked extracts were dried ($MgSO_4$), filtered and concentrated under reduced pressure.

Dry Sorbsil M60 silica (75 g) was added to the concentrated extract (50 ml), and the slurry was dried under reduced pressure. The solid remaining was applied to a column (140x3.5 cm) of dry Sorbsil M60. Elution with ethyl acetate:toluene (1:1 v/v) yielded (II) (fraction between 350 and 2150 ml). (I) was then eluted with ethyl acetate (2600 ml).

b) Purification of 2-hydroxymethyl-clavam

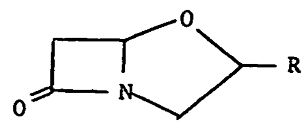
The ethyl acetate containing (I) was concentrated under reduced pressure and applied to a column (60× 6cm) of Sephadex LH20 packed in ethyl acetate. The column was eluted with ethyl acetate. Fractions between 1050 ml and 1225 ml yielded (II), which was mixed with the fraction containing (II) from the previous column, and fractions between 1825 ml and 2400 ml yielded (I). A sample of the eluate containing (I) was dried to an oil (E^1_1 after 30 min. in 0.1N NaOH=906).

c) Purification of 2-formyloxy-clavam

The combined eluates from (a) and (b) containing (II) were concentrated under reduced pressure and the concentrate was applied to a column (60x6 cm) of Sephadex LH20 packed in ethyl acetate. The column was eluted with ethyl acetate, fractions between 1025 ml and 1350 ml yielding (II). A sample of the eluate containing (II) was dried to an oil (E^1_1 after 60 min. in 0.1N NaOH=646).

WHAT WE CLAIM IS:

1. A compound of the formula (I)



(I)

wherein R represents a hydroxymethyl group, said compound in deuteroacetone exhibiting the 100 MHz proton n.m.r. τ values shown in Table I herein, and the corresponding compounds in which R is a formyloxymethyl group or a carboxyl or esterified carboxyl group or, in the case where R represents a carboxyl group, salts thereof.

2. The compound as claimed in claim 1 wherein R represents a hydroxymethyl group.

3. The compound as claimed in claim 1 wherein R represents a formyloxymethyl group.

4. The compound of claim 2 which has a negative molecular rotation $[M]_D^{23}$ in dimethyl sulphoxide of −166° or the formic acid ester thereof.

5. The compound as claimed in claim 3, a deuterochloroform solution of which has τ values in the 100 MHz proton nmr spectrum as shown in Table 2 herein.

6. A compound as claimed in claim 1 wherein R represents a carboxyl or esterified carboxyl group, or when R is a carboxyl group, salts thereof.

7. The acid as claimed in claim 6, the diphenylmethyl ester of which in a deuterochloroform solution has τ values in the 100 MHz proton nmr spectrum as shown in Table 3 herein.

8. The acid as claimed in claim 6 or claim 7, the diphenylethyl ester of which has a negative molecular rotation $[M]_D^{23}$ in dimethyl sulphoxide of −352°.

9. An ester of the acid as claimed in any of claims 6 to 8, wherein R represents an esterified carboxyl group —COOR' wherein R' represents a straight or branched substituted or unsubstituted alkyl or alkenyl group having from 1—8 carbon atoms; an aralkyl group having up to 20 carbon atoms; an aryl group having up to 12 carbon atoms; or a cycloalkyl group containing up to 12 carbon atoms optionally containing one or more heteroatoms in the ring system, and unsaturation optionally being present when a heteroatom is present.

10. A compound as claimed in claim 9 wherein R' represents a C_{1-4} alkyl group.

11. A compound as claimed in claim 9 wherein R' is a methyl group.

12. A compound as claimed in claim 9 in which R' is an arylmethyl group.

13. An alkali metal, alkaline earth metal, ammonium or organic base salt of the carboxylic acid claimed in claim 6, claim 7 or claim 8.

14. The sodium, potassium, lithium and magnesium salts of the carboxylic acid claimed in claim 6, claim 7 or claim 8.

15. A pharmaceutical composition comprising an antifungal compound as claimed in any of claims 1—14 in which R is a hydroxymethyl, formyloxymethyl or esterified carboxyl group and one or more pharmaceutical carriers or excipients.

16. A composition as claimed in claim 15 in a form suitable for oral, topical, rectal, intravaginal or parenteral administration.

17. A composition as claimed in claim 15 or claim 16 in a form suitable for topical administration.

18. A composition as claimed in any of claims 15—17 containing the active compound at a concentration of from 0.1 to 95% by weight.

19. A composition for horticultural or agricultural use which comprises one or more antifungal compounds as claimed in any of claims 1—14 in which R is a hydroxymethyl, formyloxymethyl or esterified carboxyl group in association with a carrier or diluent.

20. A composition as claimed in claim 19 containing the active material at a concentration of from 0.01 to 40% by weight.

21. A composition as claimed in claim 19 or claim 20 in the form of a dust, granulate, powder, pellet, spray, smoke or mist.

22. A process for the preparation of a compound of formula (I) as claimed in claim 1 which comprises isolating the compound of formula (I) wherein R is a hydroxymethyl, formyloxymethyl or carboxyl group from a fermentation broth of a strain of *Streptomyces clavuligerus* or where an ester of formula (I) is required,

esterifying the compound of formula (I) wherein R is carboxyl either after isolation thereof or without isolation thereof followed by isolation of the ester of formula (I) so formed.

23. A process as claimed in claim 22 wherein the strain of *Streptomyces clavuligerus* is strain NRRL 3585 or a mutant thereof.

24. A process as claimed in claim 22 wherein the strain of *Streptomyces clavuligerus* is strain NCIB 11260.

25. A process as claimed in claim 22 wherein the strain of *Streptomyces clavuligerus* is strain NCIB 11261.

26. A process as claimed in any of claims 22—25 wherein said isolation includes the step of separating the compound(s) of formula (I) wherein R represents a hydroxymethyl and/or formyloxymethyl group from other compounds present therewith.

27. A process as claimed in claim 26 wherein said separation step is effected using an anion-exchange resin whereby the desired hydroxymethyl and formyloxymethyl compounds are not retained by the resin while acidic materials are retained, and recovering the hydroxymethyl and/or formyloxymethyl compounds.

28. A process as claimed in claim 27 where the anion-exchange resin is a polystyrene, polyacrylic, epoxy-polyamine, phenolic-polyamine or cross-linked dextran resin carrying amino groups.

29. A process as claimed in claim 26 wherein said separation step is effected by extraction of an aqueous solution containing the hydroxymethyl and/or formyloxymethyl compounds into a water-immiscible solvent.

30. A process as claimed in claim 29 wherein the water-immiscible solvent is an ester or alcohol.

31. A process as claimed in claim 29 or claim 30 in which the aqueous solution is in the pH range 5—8.

32. A process as claimed in claim 26 in which, for the purification of the compounds of formula (I) in which R is hydroxymethyl and/or formyloxymethyl, an aqueous solution containing these is contacted with a non-ionic resin onto which they are adsorbed while significant quantities of impurities are not retained, followed by elution of fractions containing the desired compounds.

33. A process as claimed in claim 26 in which for the purification of the compounds of formula (I) in which R is hydroxymethyl and/or formyloxymethyl an aqueous solution containing these is contacted with a non-ionic resin which does not retain them but retains significant quantities of impurities.

34. A process as claimed in any of claims 29—33 wherein said aqueous solution is clarified or unclarified fermentation broth.

35. A process as claimed in any of claims 26—34 wherein the fermentation broth is subjected to a filtration, centrifugation or adsorption-elution technique to remove unwanted broth components prior to said separation step.

36. A process as claimed in claim 35 wherein the broth is treated with adsorbent carbon, on which the compounds of formula (I) in which R represents a hydroxymethyl, formyloxymethyl or carboxyl group are retained, and the said compounds are eluted with an aqueous water-miscible organic solvent.

37. A process as claimed in any of claims 26—36 wherein the compounds of formula (I) wherein R represents a hydroxymethyl or formyloxymethyl group are separated from each other by chromatography.

38. A process as claimed in claim 31 wherein chromatography is effected on silica or an organic solvent-compatible cross-linked dextran, and elution is effected using ethyl acetate either alone or in admixture with a hydrocarbon.

39. A process as claimed in any of claims 25 to 38 in which the product isolated is the compound of formula I in which R is hydroxymethyl.

40. A process as claimed in any of claims 25 to 38 in which the product isolated is the compound of formula I in which R is formyloxymethyl.

41. A process as claimed in any of claims 22—24 wherein the compound of formula (I) wherein R represents a carboxyl group, optionally in the form of a salt thereof, is separated from impurities.

42. A process as claimed in claim 41 in which the said carboxyl compound is separated from impurities by adsorption onto an anion exchange resin and elution therefrom of fractions containing said acid.

43. A process as claimed in claim 42 in which the anion exchange resin is a resin as defined in claim 28.

44. A process as claimed in claim 42 or claim 43 wherein removal of clavulanic

acid is effected by reaction of the material containing the acid of formula (I) or a salt thereof with a lithium salt to form the lithium salt of clavulanic acid and separating said salt by fractional crystallisation or precipitation.

45. A process as claimed in claim 44 wherein the material containing the said acidic compound of formula I is adsorbed on a column and is eluted with an aqueous solution of a lithium salt or is eluted with an aqueous eluant followed by addition of a lithium salt to the eluate.

46. A process as claimed in claim 45 wherein a water-miscible organic solvent at high concentration is incorporated into the solution of the lithium salt used for the elution or into the aqueous eluate to assist said fractional crystallisation or precipitation in order to provide 70—97% by volume of said solvent.

47. A process as claimed in claim 46 wherein said water miscible organic solvent is acetone, methanol, ethanol, isopropanol or ethylene glycol, dioxan, tetrahydrofuran, dimethylformamide or dimethylsulphoxide.

48. A process as claimed in any of claims 42 to 47 in which, prior to adsorption of said carboxyl compound onto an anion exchange resin it is adsorbed onto adsorbent carbon and eluted therefrom with an aqueous water-miscible solvent.

49. A process as claimed in any of claims 44—48 wherein the compound of formula (I) wherein R represents a carboxyl group is isolated from the mother liquor remaining after said fractional precipitation or crystallisation by solvent extraction at acidic pH or by ion-pair extraction.

50. A process as claimed in any of claims 41 to 49 in which compound of formula (I) in which R is an esterified carboxyl group is formed and the compound in which R is a carboxyl group is obtained therefrom by deesterification.

51. A process as claimed in claim 50 in which the acid of formula (I) obtained is reesterified.

52. A process as claimed in any of claims 41 to 49 in which the acid of formula (I) in which R is a carboxyl group is esterified without isolation of said acid.

53. A process as claimed in any of claims 41—52 wherein a compound of formula (I) wherein R represents a carboxyl group is esterified by treatment with a diazoalkene or diazoaralkane.

54. A process as claimed in any of claims 41—52 wherein a salt of a compound of formula (I) in which R is carboxyl is esterified by reaction with reactive derivative of an alcohol.

55. A process as claimed in claim 52 in which esterification is effected on impure material of formula (I) where R is carboxyl and the ester so formed is purified by chromatography on silica.

56. A process as claimed in claim 22 substantially as hereinbefore described.

57. A process as claimed in claim 22 substantially as hereinbefore described with reference to the Examples.

58. A compound of formula (I) as shown in claim 1 wherein R is a hydroxymethyl, formyloxymethyl, carboxyl group or esterified carboxyl group or, when R is a carboxyl group, a salt thereof whenever prepared by a process as claimed in any of claims 22—57.

59. The compound of claim 2 substantially free of any isomeric material.

60. The compound of claim 3 substantially free of any isomeric material.

61. A compound of claim 6 substantially free of any isomeric material.

62. The compound of claim 2 whenever produced by the fermentation of *Streptomyces clavuligerus*.

63. The compound of claim 3 whenever produced by the fermentation of *Streptomyces clavuligerus*.

64. A compound of claim 6 wherein the compound in which R represents a carboxyl group is produced by the fermentation of *Streptomyces clavuligerus*, together with esters or salts thereof.

65. The compound of claim 2 substantially free from other fermentation-derived materials.

66. The compound of claim 3 substantially free from other fermentation-derived material.

67. A compound of claim 6 wherein R represents a carboxyl group substantially free from other fermentation-derived material.

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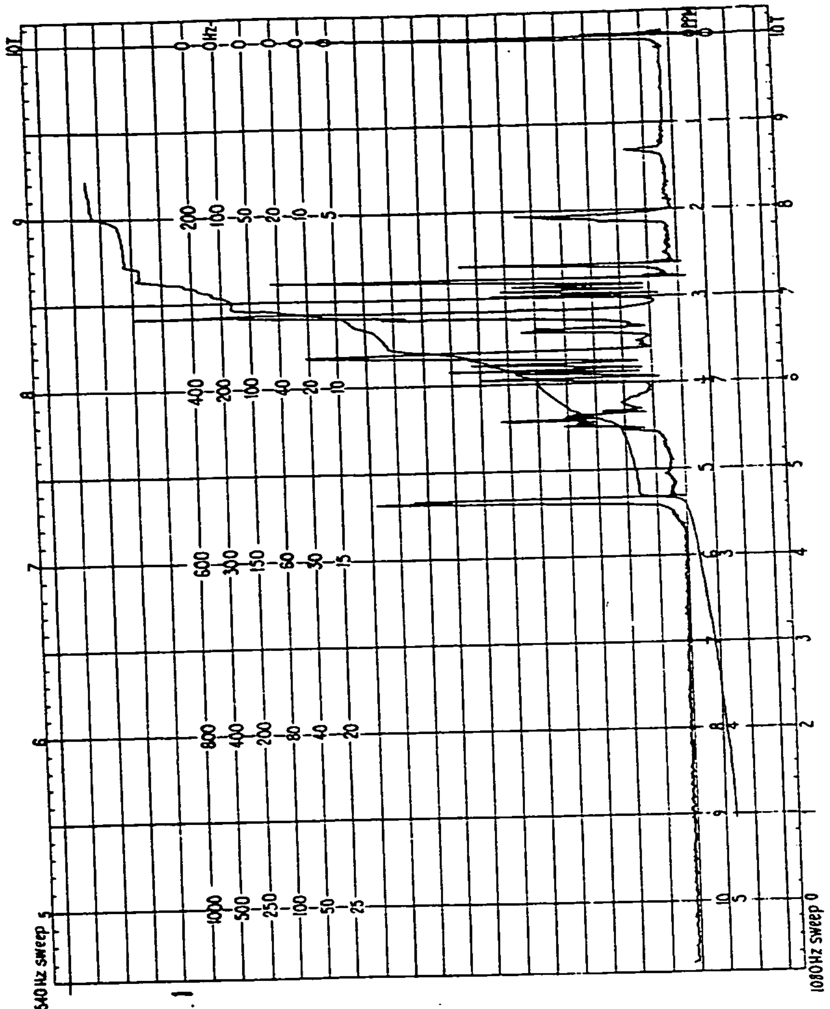

FIG. 1

1585661 COMPLETE SPECIFICATION

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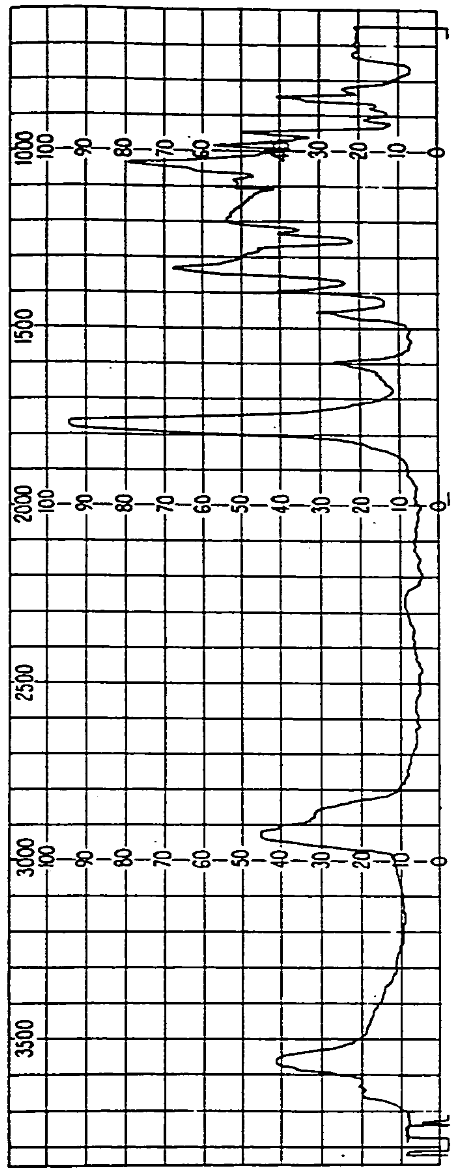

FIG. 2

1585661 COMPLETE SPECIFICATION

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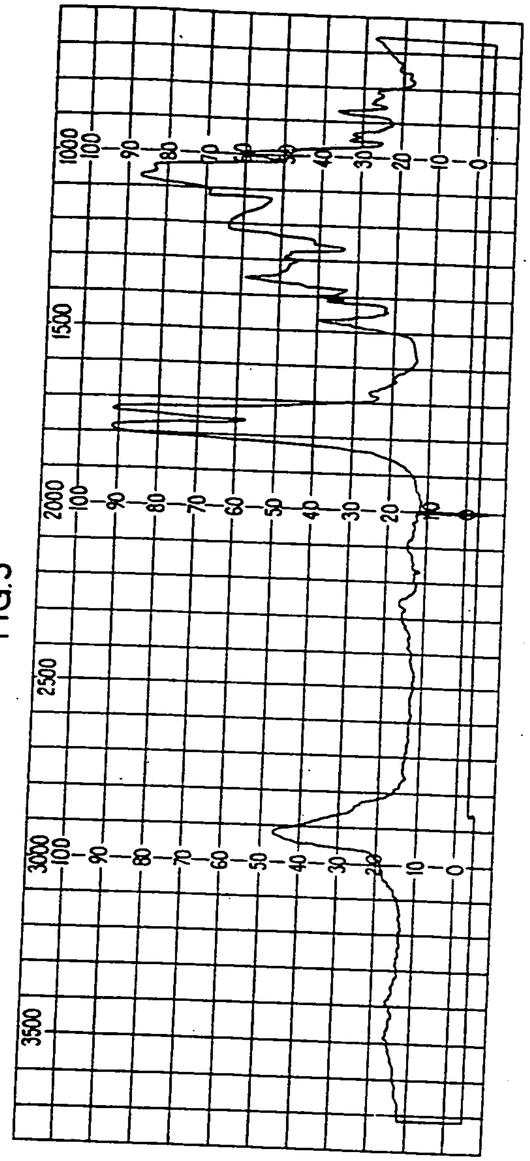

FIG.3

1585661 COMPLETE SPECIFICATION

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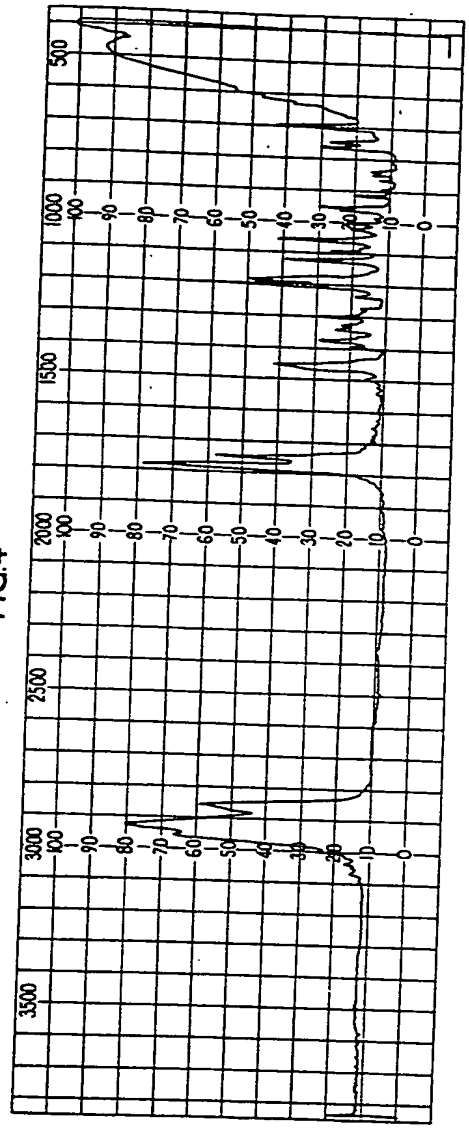

FIG.4

1585661 COMPLETE SPECIFICATION

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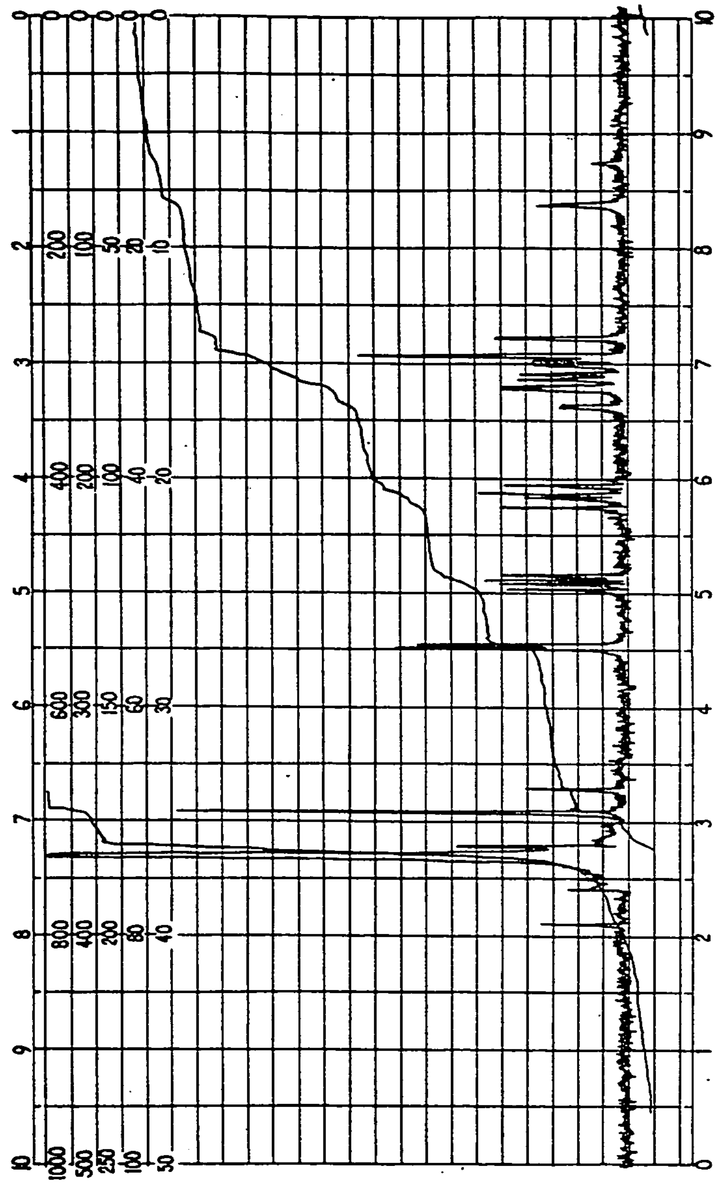
FIG.5



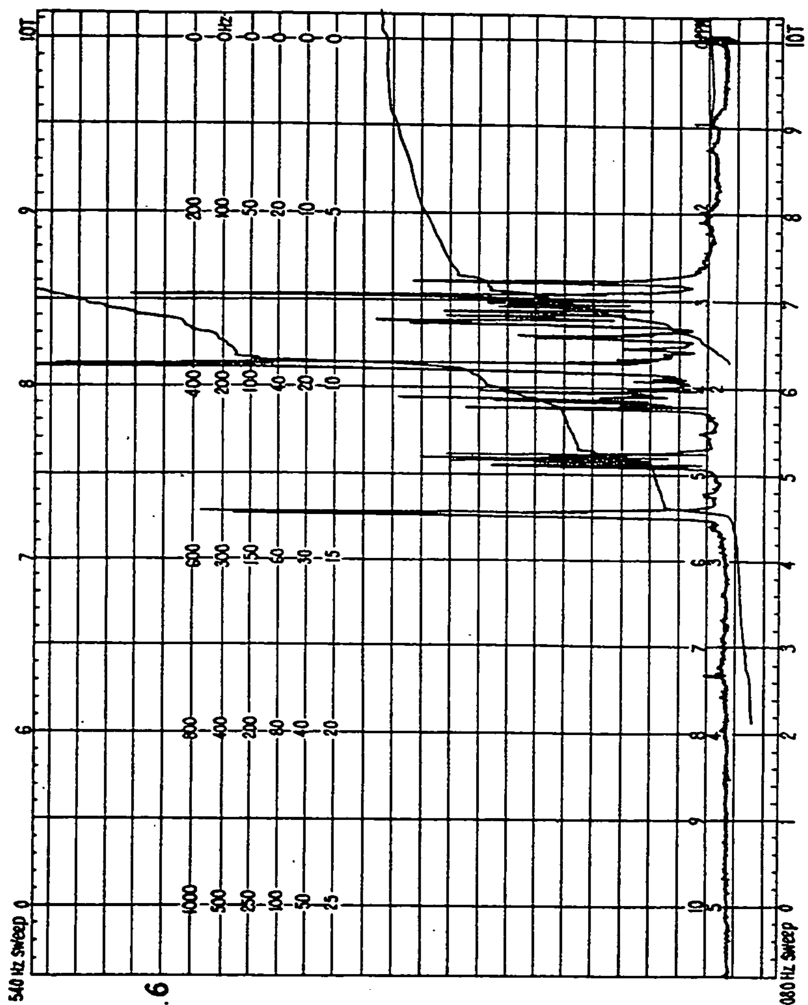


FIG.6